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RANDOX LABORATORIES LTD.
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Patents ADP number *(if you know it)* 07420268001

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

4. Title of the invention — MOLECULAR MARKER

5. Name of your agent *(if you have one)* — Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
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For the applicant
Gill Jennings & Every

Signature J John Jappy Date 10/11/03

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MOLECULAR MARKER

Field of the Invention

This invention relates to the detection of the presence of or the risk of cancer, in particular breast cancer.

Background of the Invention

There are over 1 million cases of breast cancer per year on a global basis, of which around 0.5 million are in the US, 40,000 are in the UK and nearly 2,000 in Ireland. It is the leading cause of cancer deaths among women. Although the overall incidence of the disease is increasing within the western world, wider screening and improved treatments have led to a gradual decline in the fatality rate of about 1% per year since 1991. Patients diagnosed with early breast cancer have greater than a 90% 5 year relative survival rate, as compared to 20% for patients diagnosed with distally metastasised breast cancer. Nonetheless, there is no definitive early-stage screening test for breast cancer, diagnosis currently being made on the results of mammography and fine needle biopsy. Mammography has its limitations, with over 80% of suspicious results being false positives and 10-15% of women with breast cancer providing false negative results. Often the tumour has reached a late stage in development before detection, reducing the chances of survival for the patient and increasing the cost of treatment and management for the healthcare system. More sensitive methods are required to detect small (<2 cm diameter) early stage *in-situ* carcinomas of the breast, to reduce patient mortality. In addition to early detection, there remain serious problems in classifying the disease as malignant or benign, in the staging of known cancers and in differentiating between tumour types. Finally, there is a need to monitor ongoing treatment effects and to identify patients becoming resistant to particular therapies. Such detection processes are further complicated, as the mammary gland is one of the few organs that undergo striking morphological and functional changes during adult life, particularly during pregnancy, lactation and involution, potentially leading to changes in the molecular signature of the same mammary gland over time.

Diagnosis of disease is often made by the careful examination of the relative levels of a small number of biological markers. Despite recent advances, the contribution of the current biomarkers to patient care and clinical outcome is limited. This is due to the low diagnostic sensitivity and disease specificity of the existing markers. Some molecular biomarkers, however, are being used routinely in disease diagnosis, for example prostate specific antigen in prostate cancer screening, and new candidate markers are being discovered at an increasing rate (Pritzker, 2002). It is becoming accepted that the use of a panel of well-validated biomarkers would enhance the positive predictive value of a test and minimize false positives or false negatives (Srinivas et al., 2002). In addition, there is now growing interest in neural networks, which show the promise of combining weak but independent information from various biomarkers to produce a prognostic/predictive index that is more informative than each biomarker alone (Yousef et al., 2002).

As more molecular information is collated, diseases such as breast cancer are being sub-divided according to genetic signatures linked to patient outcome, providing valuable information for the clinician. Emerging novel technologies in molecular medicine have already demonstrated their power in discriminating between disease sub-types that are not recognisable by traditional pathological criteria (Sorlie et al., 2001) and in identifying specific genetic events involved in cancer progression (Srinivas et al., 2002). Further issues need to be addressed in parallel, relating to the efficacy of biomarkers between genders and races, thus large scale screening of a diverse population is a necessity.

The management of breast cancer could be improved by the use of new markers normally expressed only in the breast but found elsewhere in the body, as a result of the disease. Predictors of the activity of the disease would also have valuable utility in the management of the disease, especially those that

(i) isolating a sample of the patient's genome; and

(ii) detecting the presence or expression of the gene characterised by the nucleotide sequence identified as SEQ ID No. 1, wherein the presence or expression of the gene indicates the presence of or the risk of cancer.

According to a second aspect of the invention, an isolated polynucleotide comprises the nucleotide sequence identified herein as SEQ ID No. 1, or its complement, or a polynucleotide of at least 15 consecutive nucleotides that hybridises to the sequence (or its complement) under stringent hybridising conditions.

According to a third aspect of the present invention, an isolated peptide comprises the sequence identified herein as SEQ ID No.2, or a fragment thereof of at least 10 consecutive amino acid residues.

According to a fourth aspect of the invention, an antibody has an affinity of at least 10^{-6}M for a peptide as defined above.

According to a fifth aspect of the invention, a polynucleotide that hybridises to or otherwise inhibits the expression of an endogenous DD20 gene, is used in the manufacture of a medicament for the treatment of cancer, in particular breast cancer.

Description of the Invention

The present invention is based on the identification of a gene that is expressed in a patient suffering cancer, in particular breast, uterus or testicular cancer. Identification of the gene (or its expressed product) in a sample obtained from a patient indicates the presence of or the risk of cancer in the patient.

The invention further relates to reagents such as polypeptide sequences, useful for detecting, diagnosing, monitoring, prognosticating, preventing, imaging, treating or determining a pre-disposition to cancer.

The methods to carry out the diagnosis can involve the synthesis of cDNA from mRNA in a test sample, amplifying as appropriate portions of the cDNA corresponding to the gene or a fragment thereof and detecting the product as an indication of the presence of the disease in that tissue, or detecting translation products of the mRNAs comprising gene sequences as an indication of the

presence of the disease.

Useful reagents include polypeptides or fragment(s) thereof which may be useful in diagnostic methods such as RT-PCR, PCR or hybridisation assays of mRNA extracted from biopsied tissue, blood or other test samples; or proteins which are the translation products of such mRNAs; or antibodies directed against these proteins. These assays also include methods for detecting the gene products (proteins) in light of possible post-translational modifications that can occur in the body, including interactions with molecules such as co-factors, inhibitors, activators and other proteins in the formation of sub-unit complexes.

The gene associated with cancer, is characterised by the polynucleotide shown as SEQ ID No. 1. The expressed product of the gene is identified herein by SEQ ID No. 2. Identification of the gene or its expressed product may be carried out using techniques known for the detection or characterisation of polynucleotides or polypeptides. For example, isolated genetic material from a patient can be probed using short oligonucleotides that hybridise specifically to the target gene. The oligonucleotide probes may be detectably labelled, for example with a fluorophore, so that, upon hybridisation with the target gene, the probes can be detected. Alternatively, the gene, or parts thereof, may be amplified using the polymerase chain reaction, with the products being identified, again using labelled oligonucleotides.

Diagnostic assays incorporating this gene, or associated protein or antibodies will include, but are not limited to:

Polymerase chain reaction (PCR)

Reverse transcription PCR

Real-time PCR

In-Situ hybridisation

Southern dot blots

Protein, antigen or antibody arrays on solid supports such as glass or ceramics, useful in binding studies.

Small interfering RNA functional assays.

All of the above techniques are well known to those in the art.

The present invention is also concerned with isolated polynucleotides that comprise the sequence identified as SEQ ID No. 1, or its complement, or fragments thereof that comprise at least 15 consecutive nucleotides, preferably 30 nucleotides, more preferably at least 50 nucleotides. Polynucleotides that hybridise to a polynucleotide as defined above, are also within the scope of the invention. Hybridisation will usually be carried out under stringent conditions. Stringent hybridising conditions are known to the skilled person, and are chosen to reduce the possibility of non-complementary hybridisation. Examples of suitable conditions are disclosed in Nucleic Acid Hybridisation. A Practical Approach (B.D. Hames and S.J. Higgins, editors IRL Press, 1985). More specifically, stringent hybridisation conditions include overnight incubation at 42°C in a solution comprising: 50% formamide, 5 x SSC (150 mM NaCL, 15 mM trisodium citrate), 50 mM sodium phosphate (ph7.6), 5 x Denhardt's solution, 10% dextran sulphate and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing in 0.1 x SSC at about 65°C.

The identification of the DD20 gene also permits therapies to be developed, with the gene being a target for therapeutic molecules. For example, there are now many known molecules which have been developed for gene therapy, to target and prevent the expression of a specific gene. One particular molecule is a small interfering RNA (siRNA), which suppresses the expression of a specific target protein by stimulating the degradation of the target mRNA. Other synthetic oligonucleotides are also known which can bind to a gene of interest (or its regulatory elements) to modify expression. Peptide nucleic acids (PNAs) in association with DNA (PNA-DNA chimeras) have also been shown to exhibit strong decoy activity, to alter the expression of the gene of interest.

The present invention also includes antibodies raised against a peptide of the invention. The antibodies will usually have an affinity for the peptide of at least 10^{-6}M, more preferably, 10^{-9}M and most preferably at least 10^{-11}M. The

antibody may be of any suitable type, including monoclonal or polyclonal. Assay kits for determining the presence of the peptide antigen in a test sample are also included. In one embodiment, the assay kit comprises a container with an antibody, which specifically binds to the antigen, wherein the antigen comprises at least one epitope encoded by the DD20 gene. These kits can further comprise containers with useful tools for collecting test samples, such as blood, saliva, urine and stool. Such tools include lancets and absorbent paper or cloth for collecting and stabilising blood, swabs for collecting and stabilising saliva, cups for collecting and stabilising urine and stool samples. The antibody can be attached to a solid phase, such as glass or a ceramic surface.

Detection of antibodies that specifically bind to the antigen in a test sample suspected of containing these antibodies may also be carried out. This detection method comprises contacting the test sample with a polypeptide which contains at least one epitope of the gene. Contacting is performed for a time and under conditions sufficient to allow antigen/antibody complexes to form. The method further entails detecting complexes, which contain the polypeptide. The polypeptide complex can be produced recombinantly or synthetically or be purified from natural sources.

In a separate embodiment of the invention, antibodies, or fragments thereof, against the antigen can be used for the detection of image localisation of the antigen in a patient for the purpose of detecting or diagnosing the disease or condition. Such antibodies can be monoclonal or polyclonal, or made by molecular biology techniques and can be labelled with a variety of detectable agents, including, but not limited to radioisotopes.

In a further embodiment, antibodies or fragments thereof, whether monoclonal or polyclonal or made by molecular biology techniques, can be used as therapeutics for the treatment of diseases characterised by the expression of

molecule that has an antigen-binding region and includes, but is not limited to, antibody fragments such as single domain antibodies (DABS), Fv, scFv etc. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art.

If desired, the cancer screening methods of the present invention may be readily combined with other methods in order to provide an even more reliable indication of diagnosis or prognosis, thus providing a multi-marker test.

The following example illustrates the invention with reference to the accompanying drawings.

Example

A number of differentially expressed gene fragments were isolated from cDNA populations derived from matched clinical samples of breast cancer patients, using non-isotopic differential display (DDRT-PCR). One of these fragments, DD20 was revealed to be significantly up-regulated in breast tumour tissue samples from a number of donors. The expression profile of this novel molecular marker, its full length and corresponding presumed protein sequence is detailed herein.

Materials and methods.

We identified differential gene expression between matched pairs of normal mammary and tumour tissue from the same donor. Tissue samples were obtained, with full ethical approval and informed patient consent, from Pathlore, Peterborough, UK. Following the surgical removal of a tumour, one sample of the tumour tissue was collected, as was a sample from the adjacent, co-excised normal tissue. Messenger RNA was extracted and cDNA subsequently synthesised, using Dynal dT_{18}-tagged Dynabeads and Superscript II reverse transcription protocols, respectively. Differential display reverse transcription PCR (DDRT-PCR) was employed to observe differences between the gene expression profiles of these matched samples, and individual gene transcripts showing up- or down-regulation were isolated and investigated further.

First described by Liang & Pardee (1992), differential display reverse transcription PCR (DDRT-PCR) uses mRNA from two or more biological samples as templates for representative cDNA synthesis by reverse transcription, with

one of 3 possible anchor primers. Each of the 3 sub-populations was PCR-amplified using its respective anchor primer coupled with one of 80 arbitrary 13-mer primers. This number of primer combinations has been estimated to facilitate the representation of 96% of expressed genes in an mRNA population (Sturtevant, 2000). This population sub-division results in the reduction of the estimated 12,000-15,000 mRNAs expressed in eukaryotic cells to 100-150 transcripts by the end of second strand cDNA synthesis for each primer set. This facilitates the parallel electrophoretic separation and accurate visualization of matched primer sets on a polyacrylamide gel, leading to the identification of gene fragments expressed in one tissue sample but not the other.

Excision and re-amplification of fragments of interest was followed by removal of false positives through reverse Southern dot blotting. This entailed the spotting of each re-amplified fragment onto duplicate nylon membranes (Hybond N+, Amersham Pharmacia Biotech) and hybridising these with either the tumour or normal tissue cDNA population of the donor from which the fragments were derived. Those fragments confirmed as differentially expressed were then direct-sequenced, i.e. without cloning, followed by web-based database interrogation to determine if each gene was novel. Fragments not matching known genes were regarded as potentially representing novel markers for the breast cancer from which they were derived. Further screening of each transcript was performed by either semi-quantitative RT-PCR or real-time PCR, using a suite of matched cDNA populations from a number of breast tumour donors. In all cases, β-actin was used as a constitutive reference gene, for calibrating the cDNA templates and as an internal positive control during PCR. Expression of each putative novel marker gene was performed through the use of gene-specific primer sets on the calibrated templates. Full-length transcripts of the novel gene fragments, including the open reading frame (that piece of the

tissue, including brain, heart, lymphocytes, spleen, kidney, testis and muscle (obtained from Origene). The DD20 molecular marker was further tested using cDNA populations derived from a more comprehensive panel of 22 human tissue types. These are as follows:

Adrenal gland	pooled from 62 donors
Bone marrow	pooled from 7 donors
Brain, cerebellum	pooled from 24 donors
Brain, whole	pooled from one donor
Colon*	pooled from one donor
Foetal brain	pooled from 59 donors
Foetal liver	pooled from 63 donors
Heart	pooled from one donor
Kidney	pooled from one donor
Liver	pooled from one donor
Lung	pooled from one donor
Placenta	pooled from 7 donors
Prostate	pooled from 47 donors
Salivary gland	pooled from 24 donors
Skeletal muscle	pooled from 2 donors
Small intestine*	pooled from one donor
Spleen	pooled from 14 donors
Testis	pooled from 19 donors
Thymus	pooled from 9 donors
Thyroid gland	pooled from 65 donors
Trachea	pooled from ? donors
Uterus	pooled from 10 donors

Note that the majority of these samples were part of the Human Total RNA panel II (Clontech), but two samples, marked with asterisks, were obtained as tissue chunks from Pathlore (Peterborough Hospital Tissue Bank) and processed at Randox Laboratories Ltd.

In addition, assays were performed on a range of ethically approved human tumour samples, as obtained through Pathlore. cDNA representative of tumours from ovary, testis, stomach, liver, lung, bladder, colon and pancreas were tested against both β-actin and DD20 by real-time PCR.

In conjunction with novel marker expression analysis, each matched pair of breast tissues was subjected to molecular signature analysis. This entailed using a suite of primers specific to a number of pre-published breast cancer

molecular markers in semi-quantitative RT-PCR against each tissue cDNA. The relationship between each molecular marker was determined and tabulated for each sample and used as a reference, against which the novel markers could be compared. This was with the aim of sub-classifying the tumour types to enable the association of novel markers against such sub-types, increasing the power of the diagnostic marker considerably.

Results and Discussion.

Using differential display, a gene fragment, termed DD20, derived from cDNA populations of matched tissue from a breast cancer donor, was observed to have significant up-regulation in the tumour cDNA population in comparison to the corresponding normal tissue cDNA. This 187-nucleotide product (Figure 1) was confirmed as differentially expressed by reverse Southern dot blots. Sequence analysis followed by database interrogation determined that DD20 was not homologous to known genes or proteins in the EMBL and SWISSPROT databases, respectively, so was regarded as potentially novel. It was, however, 100% homologous, after removal of the poly-A tail, to a clone from chromosome 11 of the human genome.

The tumour specificity of this fragment was confirmed, using gene specific primers, by semi-quantitative PCR against the originating donors matched tissue samples. These data suggest DD20 to be a putative marker for the presence of a breast tumour (Figure 2).

To facilitate further analysis, 5'-RACE was employed to extend the fragment to include the full open reading frame (ORF) of the gene, plus any 5' non-coding sequence. Using this technique, a presumed full-length product of 427 nucleotides was derived (Figure 3), which on subsequent database interrogation, confirmed the previous homology to human chromosome 11, being 100% homologous over the full length of the sequence (427/427) (Figure 4).

reasonable homology with the small inducible cytokine A22 precursors of both mouse and human, and was of a similar size to other cytokines in the SWISSPROT Database (Figure 6). However only one disulphide bridge (as indicated by the cysteine residues) is present in DD20; whereas all cytokines contain two disulphide bridges. Furthermore, this single bridge does not conform to either of those present in the cytokines.

DD20 was further screened using semi quantitative and real-time PCR analysis on cDNA populations derived from a number of matched breast tumour tissues donated by other patients. For conventional semi-quantitative PCR, 6 matched pairs of cDNA populations were assayed, initially at 40 cycles, then at 45 cycles of amplification due to the low levels of DD20 detected (Figure 7). β-actin was used for template calibration and as a positive control for PCR. In a number of these samples, notable increased expression was observed in the tumour samples, when compared to their normal counterparts. These data confirm DD20 to be a putative molecular marker for the presence of a breast tumour.

This analysis was substantiated by the molecular signature analysis of all currently available matched breast tissue samples, as follows;

Increased in tumour	10	52.6%
Increased in normal	3	15.8%
No discernable difference	4	21.1%
No expression evident	2	10.5%
Totals	19	100%

To determine organ specificity, cDNA populations from 22 non-breast human tissues were tested, both by conventional and real-time PCR, against the DD20 primers. In addition, 8 tumour tissue samples were analysed in the same way for DD20 expression. The same samples were also tested using primers from the constitutive housekeeping gene, β-actin, as a positive control and to calibrate the templates for semi-quantitative PCR analysis. The β-actin product was strongly amplified in all cDNA populations studied, confirming that the expression can be assumed to be semi-quantitative. Results of the conventional PCRs are given in Figure 8. From the panel of 30 tissue samples, DD20

appears to be selectively expressed. In most cases, strong expression of this putative marker is limited to tissues under the influence of reproductive hormones, for example ovary, testis, uterus and placenta. Weaker expression is also noted in other organs, such as bone marrow, spleen, thymus and thyroid. Of the tumours, expression is only strongly evident in the ovary and testis, and less so in the pancreas tumour.

Although not breast-specific or tumour-specific, this molecular marker shows significantly increased expression in a number of breast tumours and may relate to a specific sub-group or a tumour stage. As such, it could be useful for sub-classification of breast tumour type. Comparison of the expression profiles of DD20 in the tissue samples against the molecular signatures may reveal associations between this marker and other pre-published breast cancer markers, which have been linked to disease classification and prognosis.

For reference, it is important to point out that DD20 compares very favourably with some of the most highly regarded "standard" breast cancer markers, such as Oestrogen receptor (ERα) and human epidermal growth factor receptor (c-ErbB-2). This is evident both in the molecular signature analysis of all matched breast cancer tissue samples, where expression is similar in both samples from the same patient in many cases and using the target-specific primers against our in-house panel of 30 cDNA populations from human normal and tumour tissue. Two examples of these, namely ERα and c-ErbB-2, are shown in Figures 9 and 10, respectively. In addition, the screening of c-ErbB-2 against a selection of our matched samples is given in Figure 11. In all cases, amplified targets have been verified by sequence analysis.

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CLAIMS

1. A method for the detection of the presence of or the risk of cancer in a patient comprising the steps of:

(i) isolating a sample of the patient's genome; and

(ii) detecting the presence or expression of the gene characterised by the nucleotide sequence identified as SEQ ID No. 1,

wherein the presence or expression of the gene indicates the presence of or the risk of cancer.

2. A method according to claim 1, wherein the genome sample is obtained from breast tissue, the uterus, testis or ovary.

3. A method according to claim 1 or claim 2, wherein the cancer is breast cancer.

4. A method according to any preceding claim, wherein detection is carried out by amplifying the gene using the polymerase enzyme.

5. An isolated polynucleotide comprising the nucleotide sequence identified herein as SEQ ID No. 1, or its complement, or a polynucleotide of at least 15 consecutive nucleotides that hybridises to the sequence (or its complement) under stringent hybridising conditions.

6. Use of a polynucleotide according to claim 5, in an *in vitro* diagnostic assay to test for the risk of cancer in a patient.

7. Use according to claim 6, wherein the cancer is breast cancer.

8. A peptide comprising the sequence identified herein as SEQ ID No. 2, or a fragment thereof of at least 10 consecutive amino acid residues.

9. An antibody having an affinity of at least 10^{-6}M for the peptide of claim 8.

10. Use of a polynucleotide that hybridises with or inhibits the expression of an endogenous gene that comprises the polynucleotide according

Figure 1. Nucleotide sequence of DD20 fragment, including the poly-A tail.

```
ccaaatctag tctaactact aactagattg actcaactca gaagtagagg tacacacatt  60
tccaagagta aatactattt acttttgtat ctgctgtttt tccacataca attaccagta 120
tttagtaaca attatgttct gtacccacaa aagcaagaaa gaatgacccc attgtcaaaa 180
aaaaaaa                                                           187
```

Figure 2. Tumour specificity of DD20, as revealed by screening the matched breast cDNA populations from the originating breast cancer donor. The housekeeping gene, B-actin was used to standardise the cDNA populations. T represents tumour tissue cDNA whereas M represents co-excised mammary tissue cDNA from the same donor.

β-actin and DD20 x 40 cycle PCR

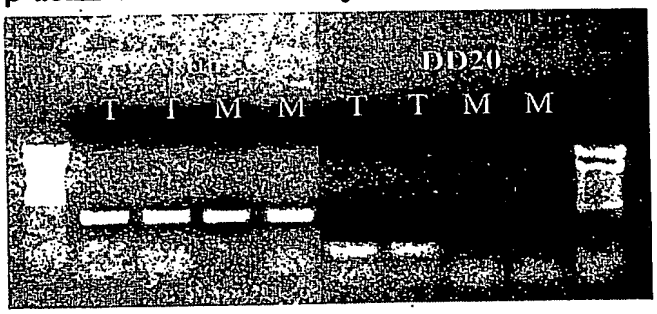


Figure 3. Nucleotide sequence of full length DD20, including the poly-A tail.

```
ctctccaaga gcttcaaact gagtaaccag caataatagt ctaccaactg ggaccaggac  60
aaaggatggt aagagattct ctctgtggta gagaatggct gaaagcaggg gatggatcag 120
caatactgaa aaaaacgttc tggtacccaa ggaaccactc taagcacaat gtacatattc 180
tatcactgga ggaattggaa gtgtgtggta cacttcaggt aacaatagca aaaacaatta 240
ccaaatctag tctaactact aactagattg actcaactca gaagtagagg tacacacatt 300
tccaagagta aatactattt acttttgtat ctgctgtttt tccacataca attaccagta 360
tttagtaaca attatgttct gtacccacaa aagcaagaaa gaatgacccc attgtcaaaa 420
aaaaaaa                                                           427
```

SEQ ID No. 1

Figure 4. Results of database searches on full length DD20.

```
>EM_HUM:AP000795 AP000795.5 Homo sapiens genomic DNA, chromosome 11
          clone:RP11-876F8, complete sequence.
       Length = 76,999

 Plus Strand HSPs:

 Score = 2090 (319.6 bits), Expect = 1.8e-86, P = 1.8e-86
 Identities = 418/418 (100%), Positives = 418/418 (100%), Strand = Plus / Plus

Query:     1 CTCTCCAAGAGCTTCAAACTGAGTAACCAGCAATAATAGTCTACCAACTGGGACCAGGAC 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 44885 CTCTCCAAGAGCTTCAAACTGAGTAACCAGCAATAATAGTCTACCAACTGGGACCAGGAC 44944

Query:    61 AAAGGATGGTAAGAGATTCTCTCTGTGGTAGAGAATGGCTGAAAGCAGGGGATGGATCAG 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 44945 AAAGGATGGTAAGAGATTCTCTCTGTGGTAGAGAATGGCTGAAAGCAGGGGATGGATCAG 45004

Query:   121 CAATACTGAAAAAAACGTTCTGGTACCCAAGGAACCACTCTAAGCACAATGTACATATTC 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 45005 CAATACTGAAAAAAACGTTCTGGTACCCAAGGAACCACTCTAAGCACAATGTACATATTC 45064

Query:   181 TATCACTGGAGGAATTGGAAGTGTGTGGTACACTTCAGGTAACAATAGCAAAAACAATTA 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 45065 TATCACTGGAGGAATTGGAAGTGTGTGGTACACTTCAGGTAACAATAGCAAAAACAATTA 45124

Query:   241 CCAAATCTAGTCTAACTACTAACTAGATTGACTCAACTCAGAAGTAGAGGTACACACATT 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 45125 CCAAATCTAGTCTAACTACTAACTAGATTGACTCAACTCAGAAGTAGAGGTACACACATT 45184

Query:   301 TCCAAGAGTAAATACTATTTACTTTTGTATCTGCTGTTTTTCCACATACAATTACCAGTA 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 45185 TCCAAGAGTAAATACTATTTACTTTTGTATCTGCTGTTTTTCCACATACAATTACCAGTA 45244

Query:   361 TTTAGTAACAATTATGTTCTGTACCCACAAAAGCAAGAAAGAATGACCCCATTGTCAA 418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 45245 TTTAGTAACAATTATGTTCTGTACCCACAAAAGCAAGAAAGAATGACCCCATTGTCAA 45302
```

Figure 5. Presumed forward open reading frames on full length DD20. Bold sequence in frame 3+ indicates putative peptide.

```
Genetic Code table used: [0] -> Standard Genetic Code
Frames: All Three Forward Frames

>_1
LSKSFKLSNQQ**STNWDQDKGW*EILSVVENG*KQGMDQQY*KKRSGTQGTTLSTMYIF
YHWRNWKCVVHFR*Q*QKQLPNLV*LLTRLTQLRSRGTHISKSKYYLLLYLLFFHIQLPV
FSNNYVLYPQKQERMTPLSRIRSKNPSTLGGQGGRIMRS
>_2
SPRASN*VTSNNSLPTGTRTKDGKRFSLW*RMAESRGWISNTEKNVLVPKEPL*AQCTYS
ITGGIGSVWYTSGNNSKNNYQI*SNY*LD*LNSEVEVHTFPRVNTIYFCICCFSTYNYQY
LVTIMFCTHKSKKE*PHCQE*EARIPALWEAKAGGS*GX
>_3
LQELQTE*PAIIVYQLGPGQRMVRDSLCGREWLKAGDGSAILKKTFWYPRNHSKHNVHIL
SLEELEVCGTLQVTIAKTITKSSLTTN*IDSTQK*RYTHFQE*ILFTFVSAVFPHTITSI
**QLCSVPTKARKNDPIVKNKKQESQHSGRPRRADHEV
```

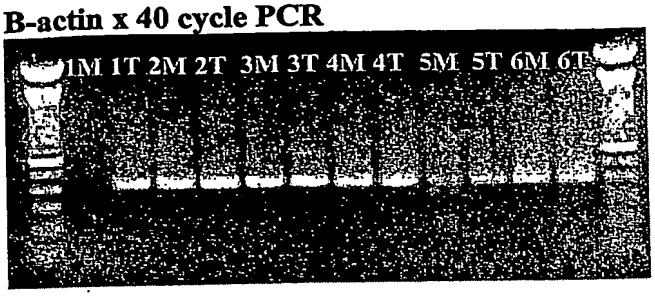
SEQ ID No.2

Figure 6. Protein database interrogation results on the full length DD20 peptide. Note the absence of cystine residues in DD20 at positions indicated in bold in the underlying cytokine sequence.

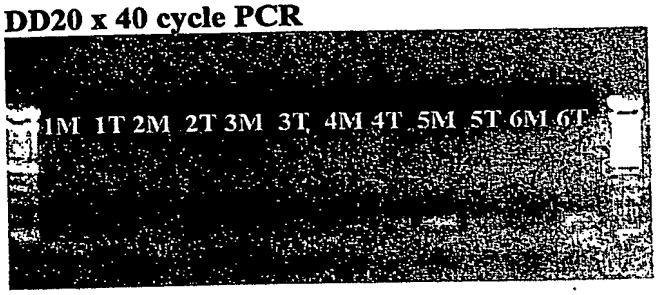
```
418 letters
Translating both strands of query sequence in all 6 reading frames

Database:  swissprot
           126,921 sequences; 46,763,021 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                    Smallest
                                                                      Sum
                                                              High  Probability
Sequences producing High-scoring Segment Pairs:               Score  P(N)      N

SW:SY22_MOUSE O88430 Small inducible cytokine A22 precurs...    82  0.0031    1
SW:SY22_HUMAN O00626 Small inducible cytokine A22 precurs...    66  0.49      1


>SW:SY22_MOUSE O88430 Small inducible cytokine A22 precursor (CCL22) (CC
          chemokine ABCD-1) (Activated B and dendritic cell-derived).
       Length = 92

  Plus Strand HSPs:

 Score = 82 (33.9 bits), Expect = 0.0031, P = 0.0031
 Identities = 20/75 (26%), Positives = 40/75 (53%), Frame = +3

Query:    30 AIIVYQLGPGQRMVRDSLCGREWLKAGDGSAILKKTFWYPRNHSKHNVHILSLEELEVCG 209
             AI     GP    V DS+C +++++    S ++K+ FW  ++  K  V +++++  ++C
Sbjct:    18 AIQTSDAGPYGANVEDSICCQDYIRHPLPSRLVKEFFWTSKSCRKPGVVLITVKNRDICA 77

Query:   210 T-LQVTIAKTITKSS 251
              QV + K + K S
Sbjct:    78 DPRQVWVKKLLHKLS 92
```

Figure 7. Expression analysis of β-actin and DD20 on cDNA populations derived from matched tumour and normal breast tissues from 6 donors. Increased expression of DD20 is evident in a number of donors.

B-actin x 40 cycle PCR



DD20 x 40 cycle PCR



DD20 x 45 cycle PCR

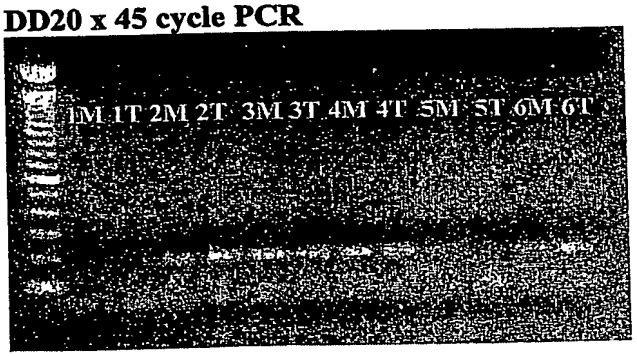
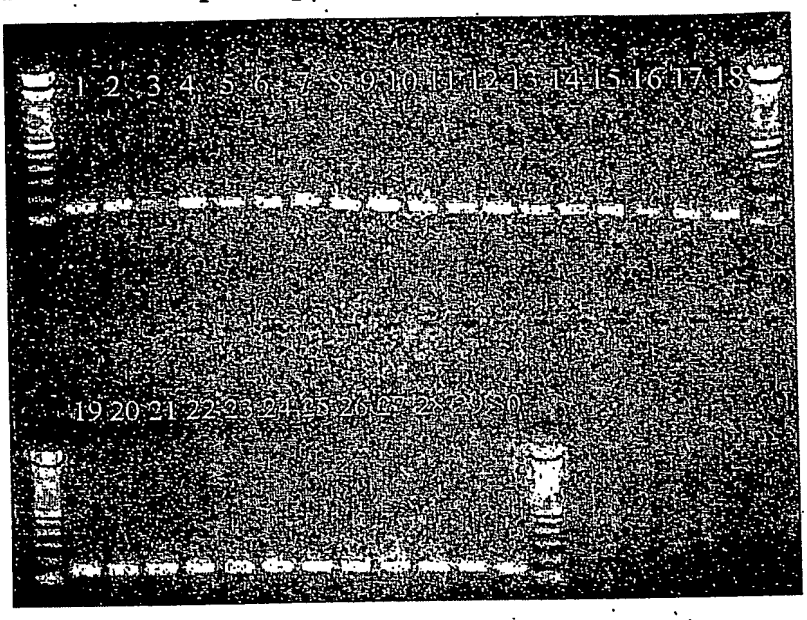
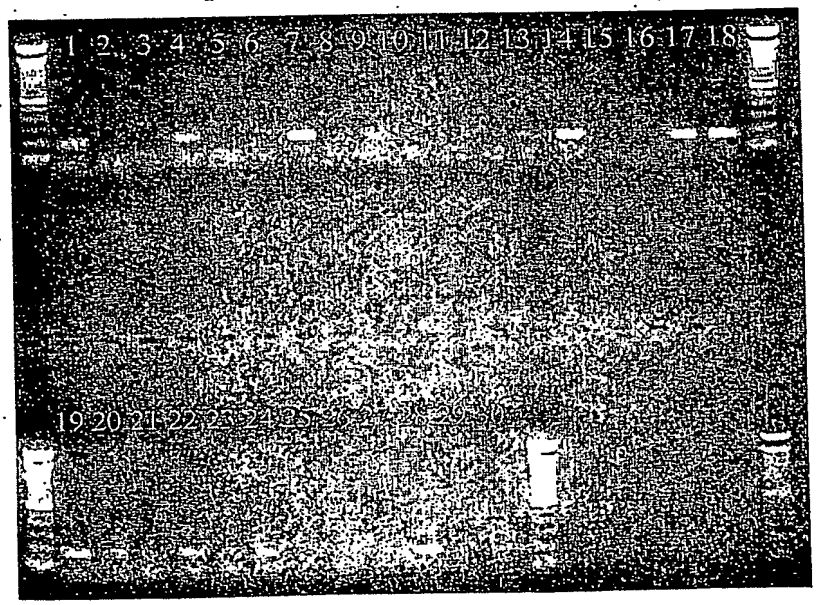


Figure 8. Conventional semi-quantitative PCR expression analysis of DD20 against a panel of 30 human tissue cDNA samples.

Human cDNA panels primed for β-actin



Human cDNA panels primed for DD20

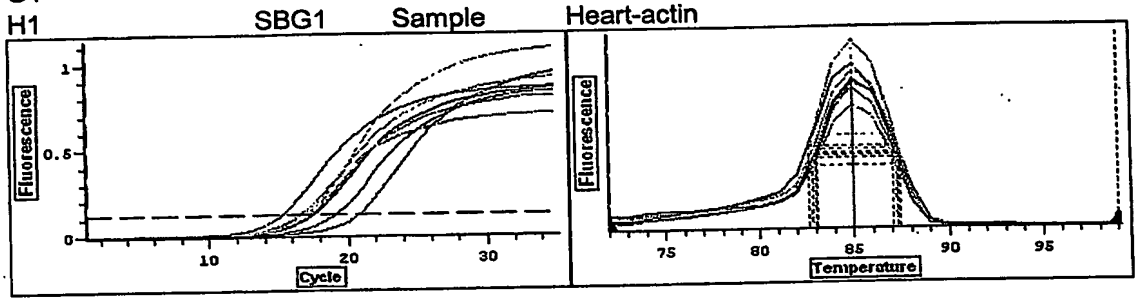


Order of cDNA templates

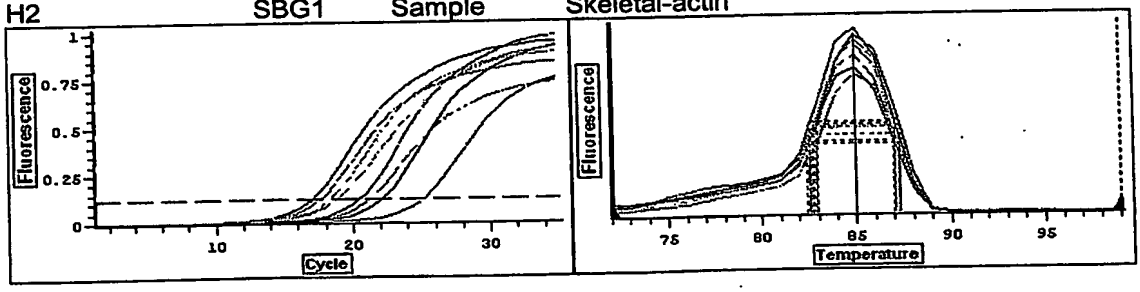
1.√	Pancreas Tumour
2.	Lung Tumour
3.	Liver Tumour
4. √	Ovary Tumour
5.	Stomach Tumour
6.	Bladder Tumour
7. √	Testis Tumour
8.	Colon Tumour
9. √	Prostate
10.	Brain-Cerebellum
11.	Kidney
12.	Heart
13.	Small Intestine
14.√	Testis
15.	Skeletal muscle
16.	Colon
17.√	Thyroid
18.√	Uterus
19.√	Placenta
20.	Trachea
21.	Foetal brain
22.√	Bone marrow
23.√	Adrenal gland
24.√	Thymus
25.	Lung
26.	Brain-Whole
27.	Foetal liver
28.√	Spleen
29.	Liver
30.	Salivary gland

Figure 9. Screening of the standard breast cancer molecular marker, ERα, against the panel of 22 human tissue and tumour cDNAs, shown as real-time data.

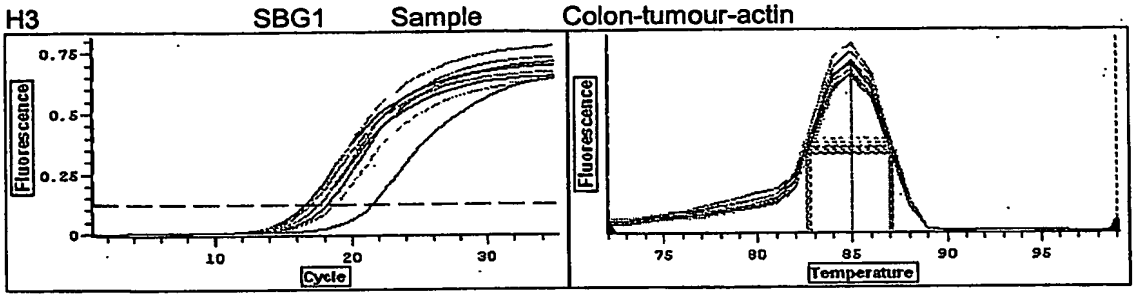
Well	Dye	Type	Label	C(T)
A1	SBG1	Sample	Adrenal-actin	19.148
B1	SBG1	Sample	Bone marrow-actin	16.557
C1	SBG1	Sample	Brain-Cereb-actin	17.542
D1	SBG1	Sample	Brain-Whole-actin	16.769
E1	SBG1	Sample	Colon-actin	15.263
F1	SBG1	Sample	Foetal Brain-actin	17.315
G1	SBG1	Sample	Foetal Liver-actin	17.149
H1	SBG1	Sample	Heart-actin	20.783



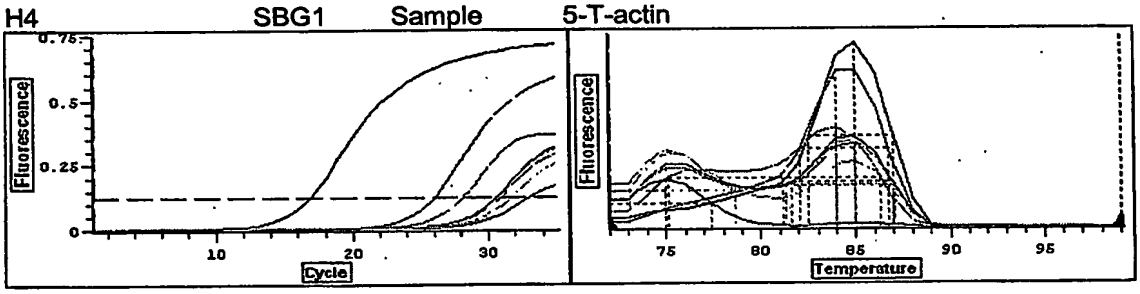
Well	Dye	Type	Label	C(T)
A2	SBG1	Sample	Intestine-small-actin	17.041
B2	SBG1	Sample	Kidney-actin	21.248
C2	SBG1	Sample	Liver-actin	22.125
D2	SBG1	Sample	Lung-actin	18.816
E2	SBG1	Sample	Placenta-actin	25.145
F2	SBG1	Sample	Prostate-actin	17.603
G2	SBG1	Sample	Salivary-actin	18.318
H2	SBG1	Sample	Skeletal-actin	20.463



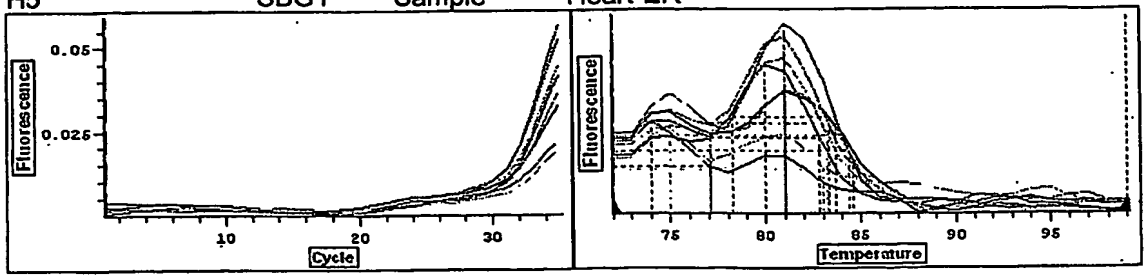
Well	Dye	Type	Label	C(T)
A3	SBG1	Sample	Spleen-actin	17.512
B3	SBG1	Sample	Testis-actin	17.514
C3	SBG1	Sample	Thymus-actin	16.559
D3	SBG1	Sample	Thyroid-actin	16.799
E3	SBG1	Sample	Trachea-actin	18.124
F3	SBG1	Sample	Uterus-actin	16.971
G3	SBG1	Sample	Bladder-tumour-actin	18.612
H3	SBG1	Sample	Colon-tumour-actin	21.413



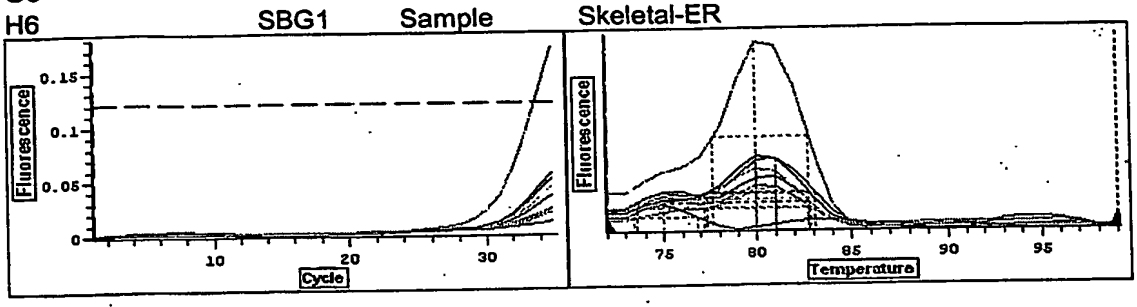
Well	Dye	Type	Label	C(T)
A4	SBG1	Sample	Liver-tumour-actin	32.978
B4	SBG1	Sample	Lung-tumour-actin	30.809
C4	SBG1	Sample	Ovary-tumour-actin	16.856
D4	SBG1	Sample	Pancreas-tumour-actin	30.537
E4	SBG1	Sample	Stomach-tumour-actin	30.590
F4	SBG1	Sample	Testis-tumour-actin	28.122
G4	SBG1	Sample	5-M-actin	31.613
H4	SBG1	Sample	5-T-actin	26.030



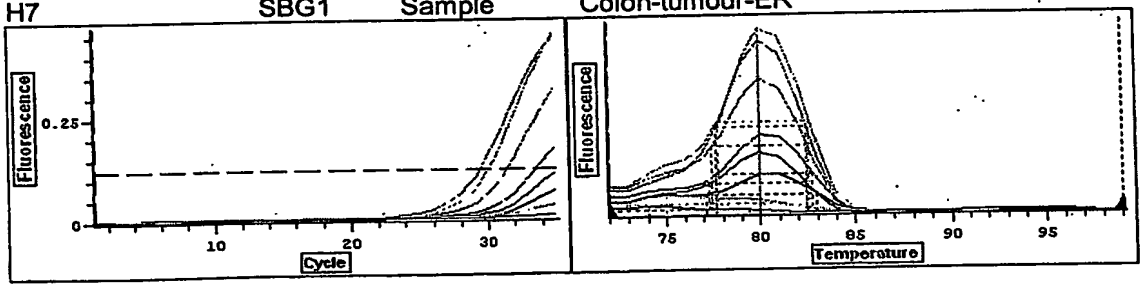
Well	Dye	Type	Label	C(T)
A5	SBG1	Sample	Adrenal-ER	None
B5	SBG1	Sample	Bone marrow-ER	None
C5	SBG1	Sample	Brain-Cereb-ER	None
D5	SBG1	Sample	Brain-Whole-ER	None
E5	SBG1	Sample	Colon-ER	None
F5	SBG1	Sample	Foetal Brain-ER	None
G5	SBG1	Sample	Foetal Liver-ER	None
H5	SBG1	Sample	Heart-ER	None



Well	Dye	Type	Label	C(T)
A6	SBG1	Sample	Intestine-small-ER	None
B6	SBG1	Sample	Kidney-ER	None
C6	SBG1	Sample	Liver-ER	None
D6	SBG1	Sample	Lung-ER	None
E6	SBG1	Sample	Placenta-ER	None
F6	SBG1	Sample	Prostate-ER	33.608
G6	SBG1	Sample	Salivary-ER	None
H6	SBG1	Sample	Skeletal-ER	None



Well	Dye	Type	Label	C(T)
A7	SBG1	Sample	Spleen-ER	None
B7	SBG1	Sample	Testis-ER	31.447
C7	SBG1	Sample	Thymus-ER	None
D7	SBG1	Sample	Thyroid-ER	30.03
E7	SBG1	Sample	Trachea-ER	33.52
F7	SBG1	Sample	Uterus-ER	29.325
G7	SBG1	Sample	Bladder-tumour-ER	None
H7	SBG1	Sample	Colon-tumour-ER	None



Well	Dye	Type	Label	C(T)
A8	SBG1	Sample	Liver-tumour-ER	None
B8	SBG1	Sample	Lung-tumour-ER	None
C8	SBG1	Sample	Ovary-tumour-ER	29.145
D8	SBG1	Sample	Pancreas-tumour-ER	None
E8	SBG1	Sample	Stomach-tumour-ER	None
F8	SBG1	Sample	Testis-tumour-ER	None
G8	SBG1	Sample	5-M-ER	None
H8	SBG1	Sample	5-T-ER	31.058

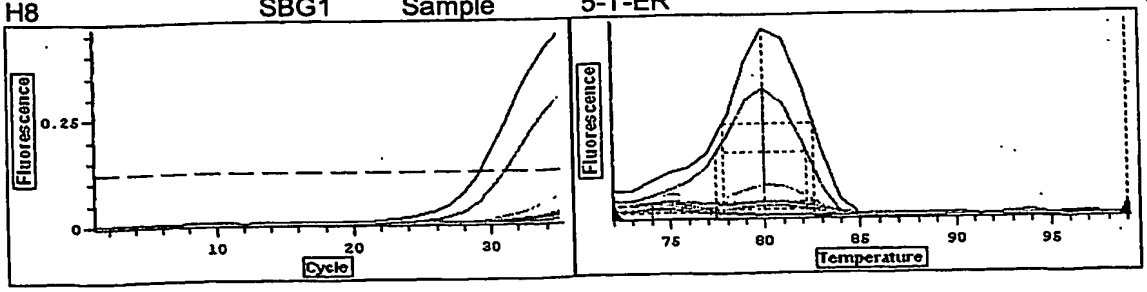
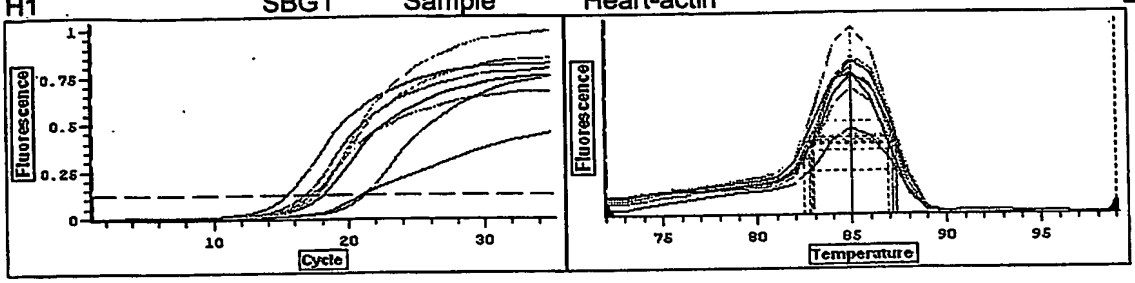
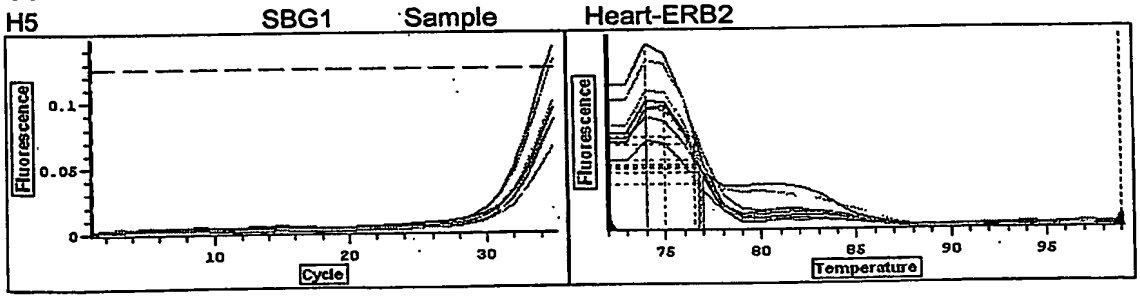


Figure 10. Screening of the standard breast cancer molecular marker, c-ErbB-2, against the panel of 22 human tissue and tumour cDNAs, shown as real-time data.

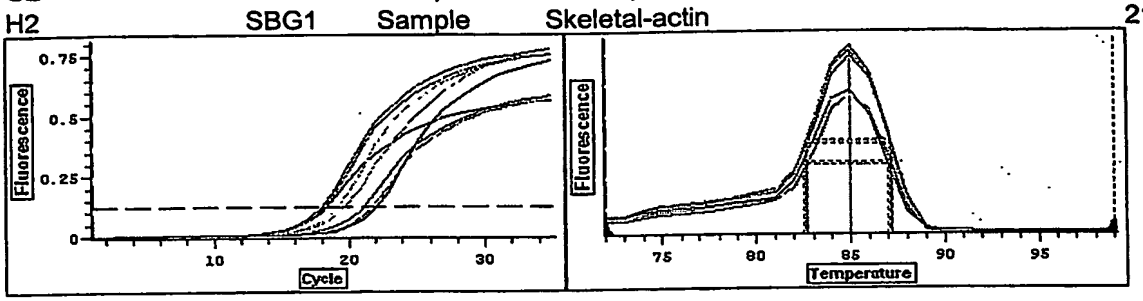
Well	Dye	Type	Label	C(T)
A1	SBG1	Sample	Adrenal-actin	21.143
B1	SBG1	Sample	Bone marrow-actin	17.294
C1	SBG1	Sample	Brain-Cereb-actin	18.104
D1	SBG1	Sample	Brain-Whole-actin	17.534
E1	SBG1	Sample	Colon-actin	15.433
F1	SBG1	Sample	Foetal Brain-actin	16.772
G1	SBG1	Sample	Foetal Liver-actin	17.541
H1	SBG1	Sample	Heart-actin	21.257



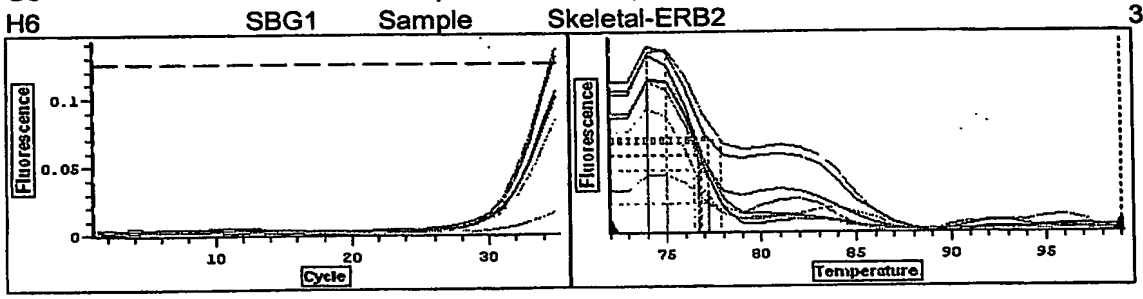
Well	Dye	Type	Label	C(T)
A5	SBG1	Sample	Adrenal-ERB2	34.416
B5	SBG1	Sample	Bone marrow-ERB2	34.745
C5	SBG1	Sample	Brain-Cereb-ERB2	None
D5	SBG1	Sample	Brain-Whole-ERB2	None
E5	SBG1	Sample	Colon-ERB2	None
F5	SBG1	Sample	Foetal Brain-ERB2	None
G5	SBG1	Sample	Foetal Liver-ERB2	None
H5	SBG1	Sample	Heart-ERB2	None



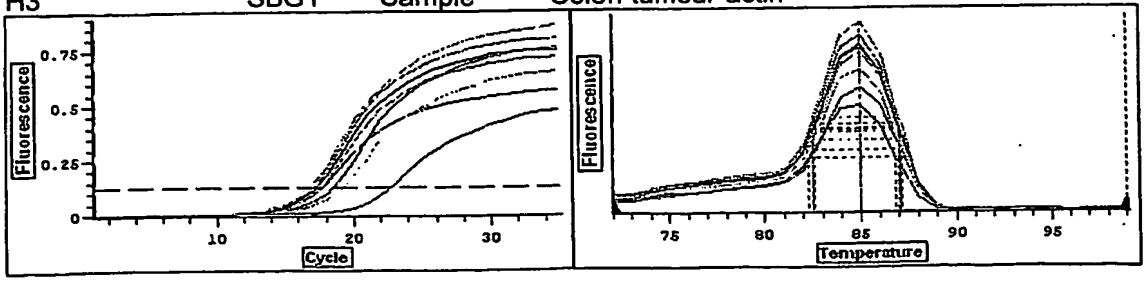
Well	Dye	Type	Label	C(T)
A2	SBG1	Sample	Intestine-small-actin	18.352
B2	SBG1	Sample	Kidney-actin	21.784
C2	SBG1	Sample	Liver-actin	22.140
D2	SBG1	Sample	Lung-actin	19.684
E2	SBG1	Sample	Placenta-actin	17.995
F2	SBG1	Sample	Prostate-actin	18.189
G2	SBG1	Sample	Salivary-actin	19.161
H2	SBG1	Sample	Skeletal-actin	21.121



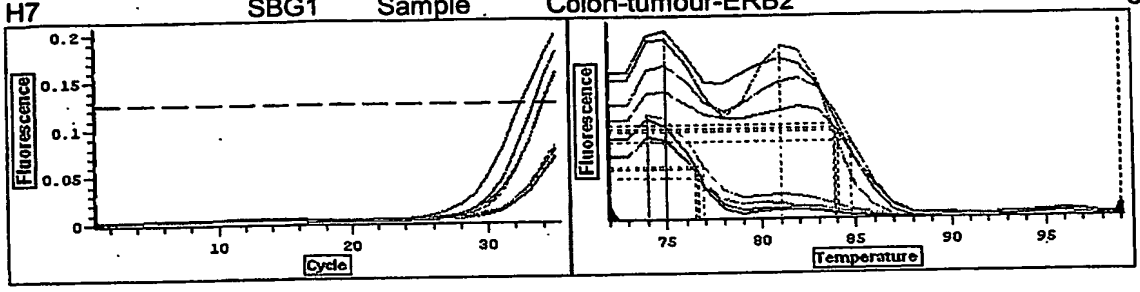
Well	Dye	Type	Label	C(T)
A6	SBG1	Sample	Intestine-small-ERB2	34.782
B6	SBG1	Sample	Kidney-ERB2	None
C6	SBG1	Sample	Liver-ERB2	None
D6	SBG1	Sample	Lung-ERB2	None
E6	SBG1	Sample	Placenta-ERB2	None
F6	SBG1	Sample	Prostate-ERB2	34.614
G6	SBG1	Sample	Salivary-ERB2	None
H6	SBG1	Sample	Skeletal-ERB2	34.794



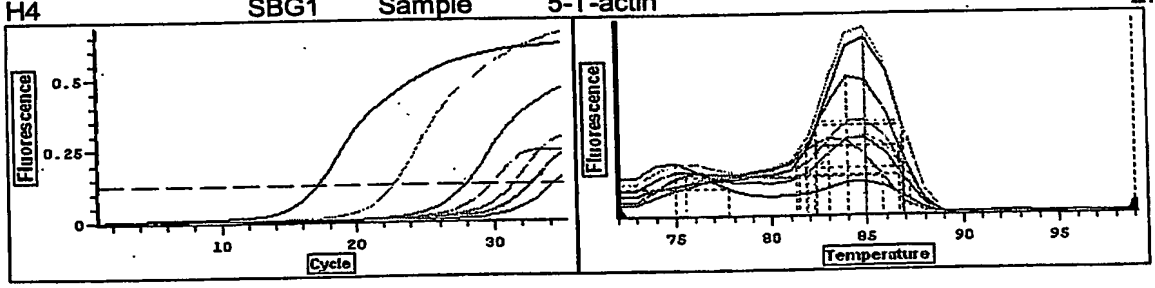
Well	Dye	Type	Label	C(T)
A3	SBG1	Sample	Spleen-actin	17.643
B3	SBG1	Sample	Testis-actin	17.527
C3	SBG1	Sample	Thymus-actin	17.235
D3	SBG1	Sample	Thyroid-actin	16.800
E3	SBG1	Sample	Trachea-actin	18.473
F3	SBG1	Sample	Uterus-actin	17.049
G3	SBG1	Sample	Bladder-tumour-actin	19.166
H3	SBG1	Sample	Colon-tumour-actin	22.747



Well	Dye	Type	Label	C(T)
A7	SBG1	Sample	Spleen-ERB2	None
B7	SBG1	Sample	Testis-ERB2	34.041
C7	SBG1	Sample	Thymus-ERB2	None
D7	SBG1	Sample	Thyroid-ERB2	None
E7	SBG1	Sample	Trachea-ERB2	33.373
F7	SBG1	Sample	Uterus-ERB2	33.958
G7	SBG1	Sample	Bladder-tumour-ERB2	None
H7	SBG1	Sample	Colon-tumour-ERB2	32.476



Well	Dye	Type	Label	C(T)
A4	SBG1	Sample	Liver-tumour-actin	34.271
B4	SBG1	Sample	Lung-tumour-actin	31.338
C4	SBG1	Sample	Ovary-tumour-actin	17.152
D4	SBG1	Sample	Pancreas-tumour-actin	22.753
E4	SBG1	Sample	Stomach-tumour-actin	32.659
F4	SBG1	Sample	Testis-tumour-actin	29.761
G4	SBG1	Sample	5-M-actin	32.761
H4	SBG1	Sample	5-T-actin	27.948



Well	Dye	Type	Label	C(T)
A8	SBG1	Sample	Liver-tumour-ERB2	34.812
B8	SBG1	Sample	Lung-tumour-ERB2	32.515
C8	SBG1	Sample	Ovary-tumour-ERB2	30.024
D8	SBG1	Sample	Pancreas-tumour-ERB2	None
E8	SBG1	Sample	Stomach-tumour-ERB2	None
F8	SBG1	Sample	Testis-tumour-ER	34.800
G8	SBG1	Sample	5-M-ERB2	None
H8	SBG1	Sample	5-T-ERB2	None

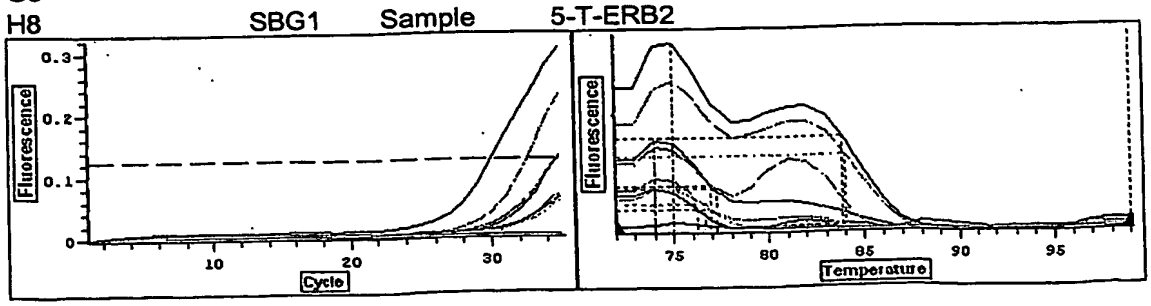
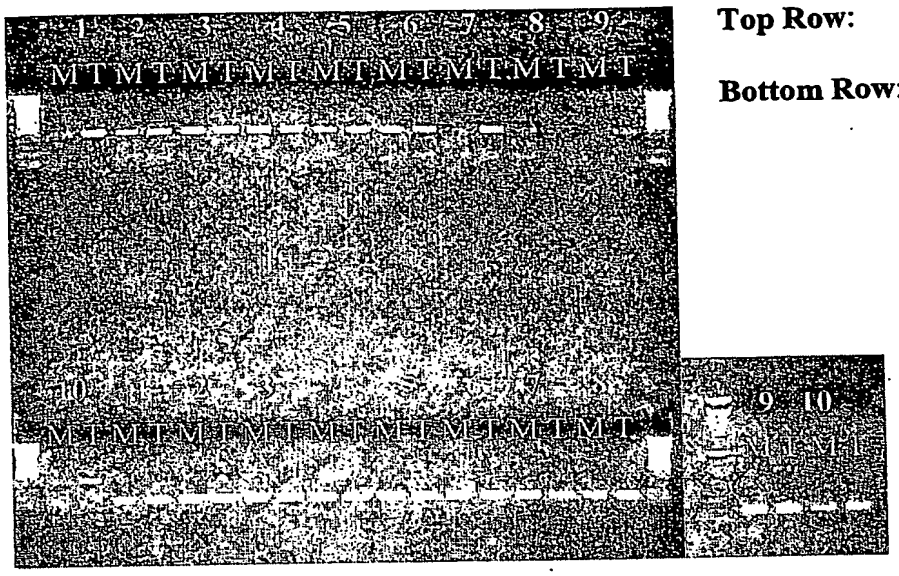


Figure 11. Screening of the standard breast cancer molecular marker, c-ErbB-2, against 10 of the matched normal and tumour breast tissue cDNAs. β-actin expression is also shown, for confirmation of template calibration and integrity. Note that increased tumour expression of this gene is only evident in 4 of the 10 matched tissues.



Top Row: c-ErbB-2

Bottom Row: β-actin

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